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14. ABSTRACT The goal of this research was to firmly establish the mechanism of androgen receptor (AR)-JunD heterodimer induction of the SSAT gene leading to oxidative stress that contributes to the development and progression of prostate cancer (PCa), and to identify small molecules that specifically inhibit this AR-JunD interaction and prevent development/progression of PCa in preclinical models. Data from this research would identify the most efficacious drug to be further developed in preclinical toxicity testing and clinical trials for PCa that fall beyond the scope of this proposal. From this research it has been established that certain sequences of the SSAT promoter are important for androgen-induced SSAT activation, and that compounds that specifically inhibit the interaction of AR and JunD can block the AR-JunD ROS-generating pathway and inhibit growth in PCa cells. Furthermore, the lead AR-JunD inhibitor showed efficacy against the castrate-resistant C4-2 PCa xenograft model. However, lack of efficacy against androgen-dependent LNCaP and TRAMPxFVB transgenic PCa models suggests its application more for treatment of CRPCa than for chemopreventive or early-stage PCa therapy. Further pre-clinical animal efficacy studies are needed to support translation of the lead compound to clinical testing. Overall the data supports AR-JunD inhibitors as a new class of agents for further research and development as new therapies for early-stage recurrent PCa patients, who have no approved therapy and represent a long unmet medical need.					
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Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	10
Reportable Outcomes.....	10
Conclusion.....	10
References.....	11
Bibliography.....	12
Personnel.....	12
Appendices.....	12

INTRODUCTION

We hypothesize that activated androgen receptor (AR) forms a heterodimer with JunD and this AR-JunD heterodimer induces SSAT gene expression leading to polyamine oxidation and consequent production of excess reactive oxygen species (ROS) in prostate cells. Excess ROS, in turn, contribute to the development and progression of prostate cancer (PCa). The purpose of this research was to firmly establish the mechanism of the induction of SSAT by the AR-JunD complex that leads to an excess ROS production in PCa cells, identify small molecules that specifically inhibit the AR-JunD interaction downstream of androgen activation of AR in this pathway, validate the activity of the inhibitors against ROS production and growth in PCa cells, and determine the chemotherapeutic/chemopreventive efficacy of the lead inhibitors against pre-clinical mouse models of PCa. Data from this research would identify the most efficacious drug to be further developed for preclinical toxicity testing and clinical trials for PCa that fall beyond the scope of this proposal. This final report summarizes work completed over the entire research period to achieve the proposed aims.

BODY

The following are the data collected with respect to tasks listed in our statement of work (SOW):

Task 1. Establish the mechanistic pathway for androgen-induced SSAT expression in CaP cells:

- 1.1. Create mutant SSAT promoter-luciferase reporter constructs.*
- 1.2. Transiently transfect mutant constructs into cells and compare 1) SSAT promoter activity by luciferase reporter assay and 2) reduction in androgen-induced ROS production by DCFH dye oxidation assay.*
- 1.3. Perform ChIP assay to identify the AR-JunD binding sequence in most and least effective mutants.*

Cell culture studies on LNCaP cells transiently transfected with SSAT promoter-luciferase reporter constructs, including three differently truncated promoter constructs and the full length promoter construct, showed enhancement of SSAT promoter activity following androgen stimulation for all but the truncated promoter with a 659 bp lead sequence (Year 1 Report, Fig.1 and Table 1). The data suggested a 3-dimensional structure of the promoter that may hide certain sections in the SSAT promoter that are important for androgen induction of SSAT. Our previously published chromatin immunoprecipitation (ChIP) data showed that a 77 bp section between 550-650 bp lead sequence is the JunD binding domain of the SSAT promoter in the presence of androgen [1]. We introduced point mutations in the AP1/JunD binding sequences within the SSAT promoter sequence and repeated the studies with these point-mutated constructs. No difference in SSAT promoter activity following androgen stimulation was observed, thus we concluded that the point mutations did not disturb JunD binding. Since the point mutations had no effect, we did not pursue the ChIP assay in cells transfected with these constructs as proposed.

Task 2. Screen a small molecule library to identify inhibitors of AR-JunD interaction.

- 2.1 Optimize the GL-reconstitution screening assay conditions using the “hit” obtained from the partial screening assay performed in preliminary studies.*
- 2.2. Perform the optimized screen on the 14,400 compound Maybridge HitFinder library.*
- 2.3. Eliminate false positive “hits” by performing the positive control (SMAD3-PKB) interaction screen.*

After standardizing a more efficient 384-well plate instead of 96-well plate based high throughput screen (HTS) assay as proposed, we completed our screen for new “hits” in a larger, 25,000 compound Life Chemicals library (at no extra cost), which yielded 13 small molecule drug-like compounds that specifically inhibited the AR-JunD interaction by at least 30%, and were thus advanced to Task 3. Details of the HTS and selection of AR-JunD inhibitors have been published (Appendix 1, Mehraein-Ghomi, *et al* publication in *The Prostate*).

Task 3. Select inhibitors that act downstream to AR activation and validate compounds for activity against CaP cells:

3.1 Test compounds for their ability to bind AR using an AR-LBD binding fluorescence polarization assay.

3.2 Test compounds for their ability to inhibit the translocation of AR-JunD to the cell nucleus.

3.3 Test compounds for their ability to inhibit growth of CaP cells and block androgen-induced ROS production in CaP cells.

Details of the AR-LBD binding and cell culture analyses on the compounds from the Life Chemicals library funded by this grant as well as NCI Diversity Set compounds accomplished with other funding have been published (Appendix 1, Mehraein-Ghomi, *et al* publication in *The Prostate*). We determined that 12 of the 13 compounds from the Life Chemicals library identified in Task 2 are non-antiandrogenic, and thus should act downstream to AR activation by androgen. We selected 7 of the 12 non-antiandrogenic compounds to be tested in the full series of ROS and growth cell culture studies, based on $\geq 50\%$ inhibition of the AR-JunD interaction by these 7 compounds in the HTS assay. DCF and DNA assays were used to measure the amount of ROS production and growth, respectively, in both androgen-dependent LNCaP and androgen-independent LNCaP C4-2 cell lines following our established methods [2-4], and the compounds were ranked based on efficacy against cell growth (Year 2 Report, Table 1 and Fig1). Two compounds, GWARJD10-001 and GWARJD14-001, showed significant activity against androgen-induced ROS production and AD growth in LNCaP cells as well as LNCaP C4-2 AI growth, as published (Appendix 1), and thus these compounds were prioritized for further mechanistic studies. Immunocytochemistry (ICC) studies to determine the ability of the top compounds GWARJD10-001 and GWARJD14-001 to inhibit the nuclear translocation of AR-JunD complex were performed in LNCaP cells treated with ROS-inducing levels of R1881 synthetic androgen and varying doses of compound. We established a quantitative ICC analysis using new, sophisticated technology - a Nuance™ fluorescence microscope (Caliper/PerkinElmer) equipped with spectral deconvolution and AUC integration software - available in the UWCCC TRIP lab. GWARJD10-001 showed significant activity against androgen-stimulated co-translocation of AR and JunD to LNCaP cell nuclei, while GWARJD14-001 was not effective at the same concentrations (Year 2 Report, Fig.2). However, since AR staining was less than optimal in those studies, we optimized the AR staining and fluorescence imaging with assistance from the UWCCC Experimental Pathology Laboratory to complete ICC analysis on the lead compound for publication. Using a novel ICC technique, Proximity Ligation Assay (PLA), that quantitates protein-protein interaction *in situ*, we verified the increase in AR-JunD interaction following treatment with androgen in LNCaP cells and further confirmed that this interaction of AR with JunD is significantly inhibited by lead compound GWARJD10-001, as published (Appendix 1). We also reported in the publication that this compound blocks the induction of SSAT gene expression in the AR-JunD ROS-generating pathway, as well as reduces AR transcriptional activity under androgen-deprived conditions, without affecting AR and JunD protein expression or degradation (Appendix 1). Additionally, we showed that the mechanism of growth inhibition by lead compound GWARJD10-001 likely does not involve cell kill or apoptosis, but rather involves a reduction of cell cycle regulator cyclin D1 (Appendix 1) and thus probably blocks cell cycle progression.

Task 4. Select the lead drug candidate for future clinical testing by comparing efficacy of 2 to 3 drug candidates in mouse models of CaP.

4.1 Screen potential drug candidates for oral bioavailability, pharmacokinetics and maximum tolerated dose in mice.

4.2 Determine efficacy of drug candidates against a CaP cell xenograft model in nude mice.

4.3 Determine efficacy of drug candidates against the TRAMP mouse model of CaP.

4.4 Complete a statistical comparison of the efficacy of drug candidates against both CaP animal models to select the lead drug for further pre-clinical testing and future clinical trial.

The lead compound from *in vitro* studies, GWARJD10-001, was advanced into animal studies. Solubility of the compound was determined in our standard administration vehicle, 0.9% saline. A preliminary PK study in adult male mice established that a single dose of 5 mg/kg in 6% DMSO saline by either intra venous (IV) or oral administration was well-tolerated and yielded plasma levels at 30 minutes post administration of 3.2nM for IV and .05nM for oral dosing (Year 2 Report, Table 2). Thus, the compound was determined to have oral bioavailability, and is therefore an ideal candidate for future clinical application, particularly for treatment of patients with early-stage prostate cancer. Additional PK / maximum tolerated dose (MTD) studies for a daily oral dosing regimen of GWARJD10-001 were undertaken. Studies to identify the lethal dose 10% (LD10) (to be used as MTD), established the regimen of 50 mg/kg oral dailyx5 every week (using a 60% DMSO in saline formulation to maintain drug in solution) as MTD for use in efficacy studies. Statistical analysis of the first MTD study (Figure 1), in which 1 of 3 mice in the 50mg/kg daily oralx5 weekly regimen had to be euthanized at Week 3 due to excess weight loss and was thus considered a toxic event, i.e. drug lethality, yielded an estimated LD10 of 48.2 mg/kg. The second MTD study (Figure 1), in which no additional toxic events were observed, body weights were maintained and there was no apparent organ toxicity, established the MTD at 50mg/kg.

Analysis of plasma from the additional PK and MTD study mice showed that an oral dose of 50mg/kg achieved a plasma level of 1 nM GWARJD10-001 at 2 hours post administration (Table 1), but the drug was completely cleared from plasma within 24 hours as seen in both the PK study (Table 1) as well as in MTD study mice (N=8) harvested after four weeks of treatment (20 doses of drug) at nadir timepoint, i.e., 2 days after the last treatment, that had no detectable drug in plasma.

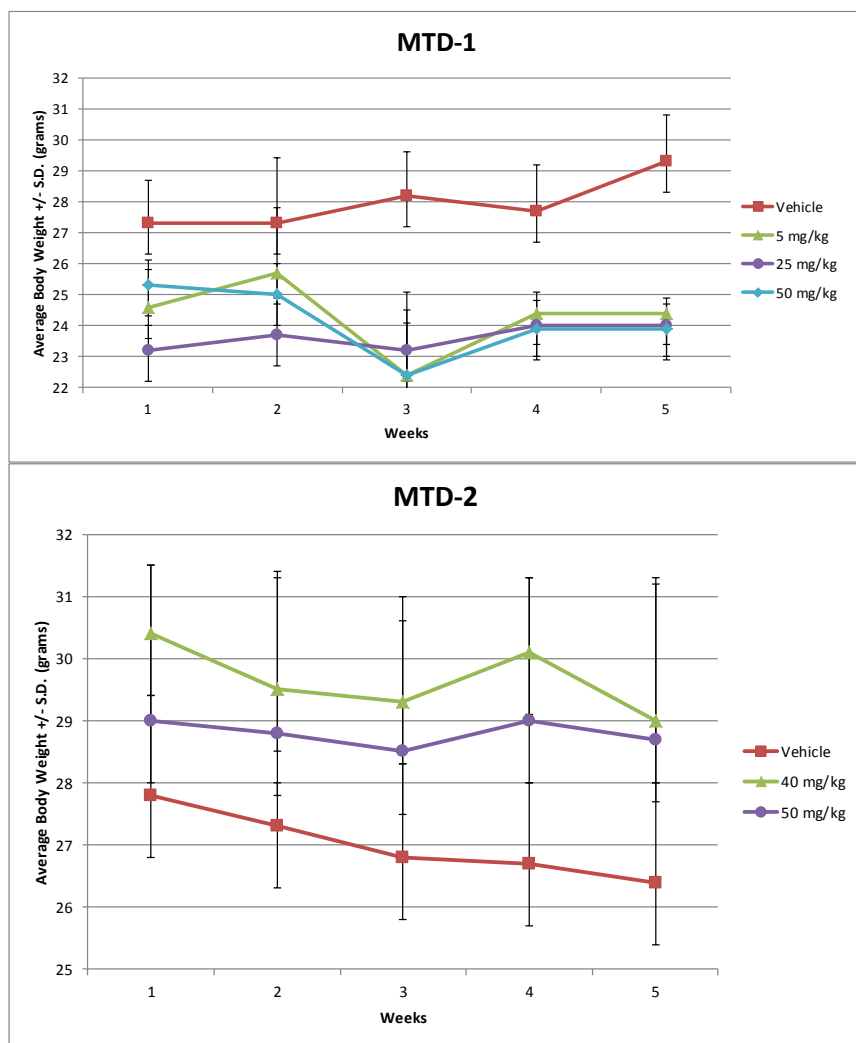


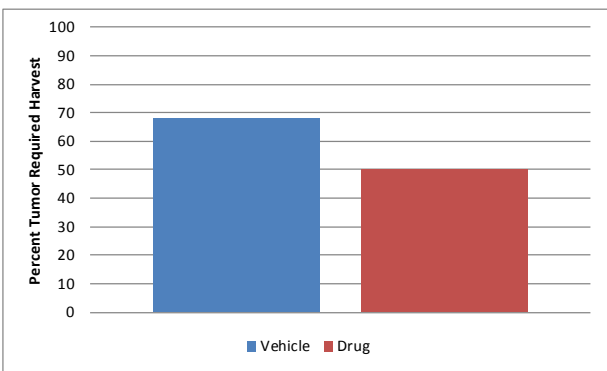
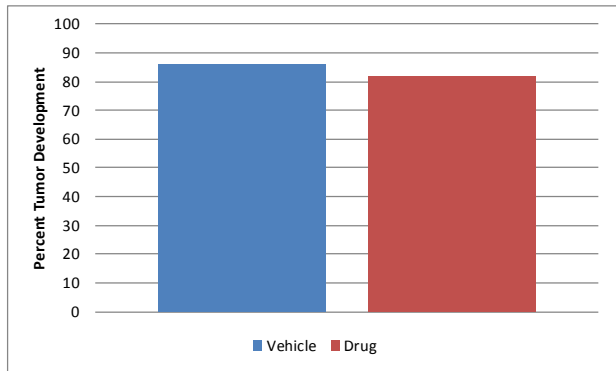
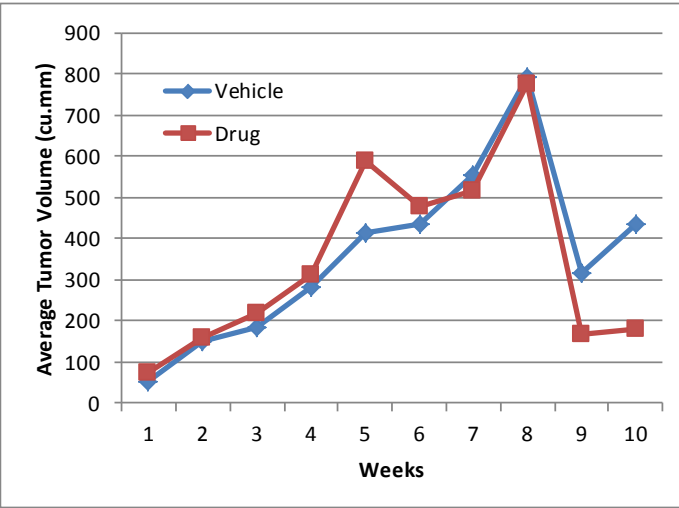
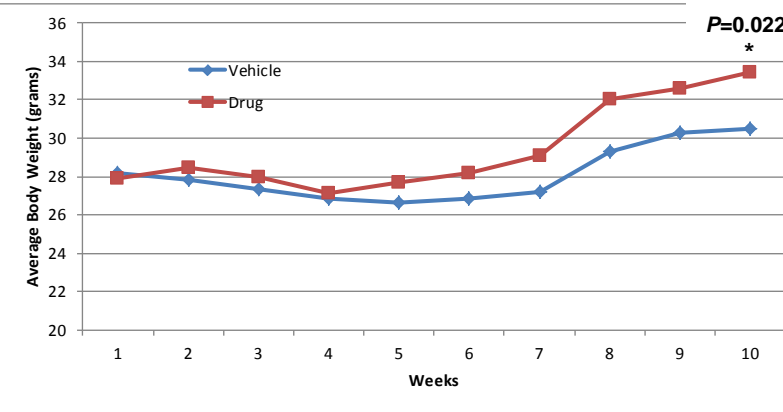
Figure 1. Maximum tolerated dose (MTD) of GWARJD10-001 in mice. Adult male mice of C57/Bl6 origin were randomized to drug or vehicle control (60%DMSO in saline) treatment groups. Treatments were administered oral daily x 5 every week for 4 weeks. The first study (MTD-1, top panel) included N=3 mice per condition and allowed for estimation of LD10 to target in the second study (MTD-2, bottom panel), which included vehicle control N=2, 40 mg/kg N=4 and 50 mg/kg N=6.

Table 1. Levels of GWARJD10-001 in plasma following single oral dose.

Mice were harvested at designated timepoints following a single dose administration of compound in 60% DMSO-saline vehicle (N=3 per condition). Plasma was extracted and LC-MS carried out by a standardized gradient of 2% acetic acid water and 2% acetonitrile.				
	<u>2 hours</u>	<u>24 hours</u>		
5 mg/kg	0.06 +/- 0.06 nM	0		
50 mg/kg	1.00 +/- 0.27 nM	0		

We completed efficacy studies against both the androgen-dependent LNCaP cells (AD LNCaP) and the variant castrate-resistant C4-2 LNCaP cells (CR C4-2) *in vivo* xenograft models of PCa. Results are shown in Figure 2A (AD LNCaP) and Figure 2B (CR C4-2). AD LNCaP model mice treated with drug had significantly higher body weights than control mice, which suggests some benefit of drug, but no benefit for tumor development or growth was observed, thus drug was overall not effective against AD LNCaP. CR C4-2 model mice treated with drug also had significantly higher body weights than control mice, and additionally had significant 4-fold reduction in tumor development and 7-fold reduction in tumor required harvest. Thus GWARJD10 showed efficacy against the CR C4-2 xenograft model.

A. Androgen-Dependent LNCaP Xenograft Model



B. Castrate-Resistant C4-2 Xenograft Model

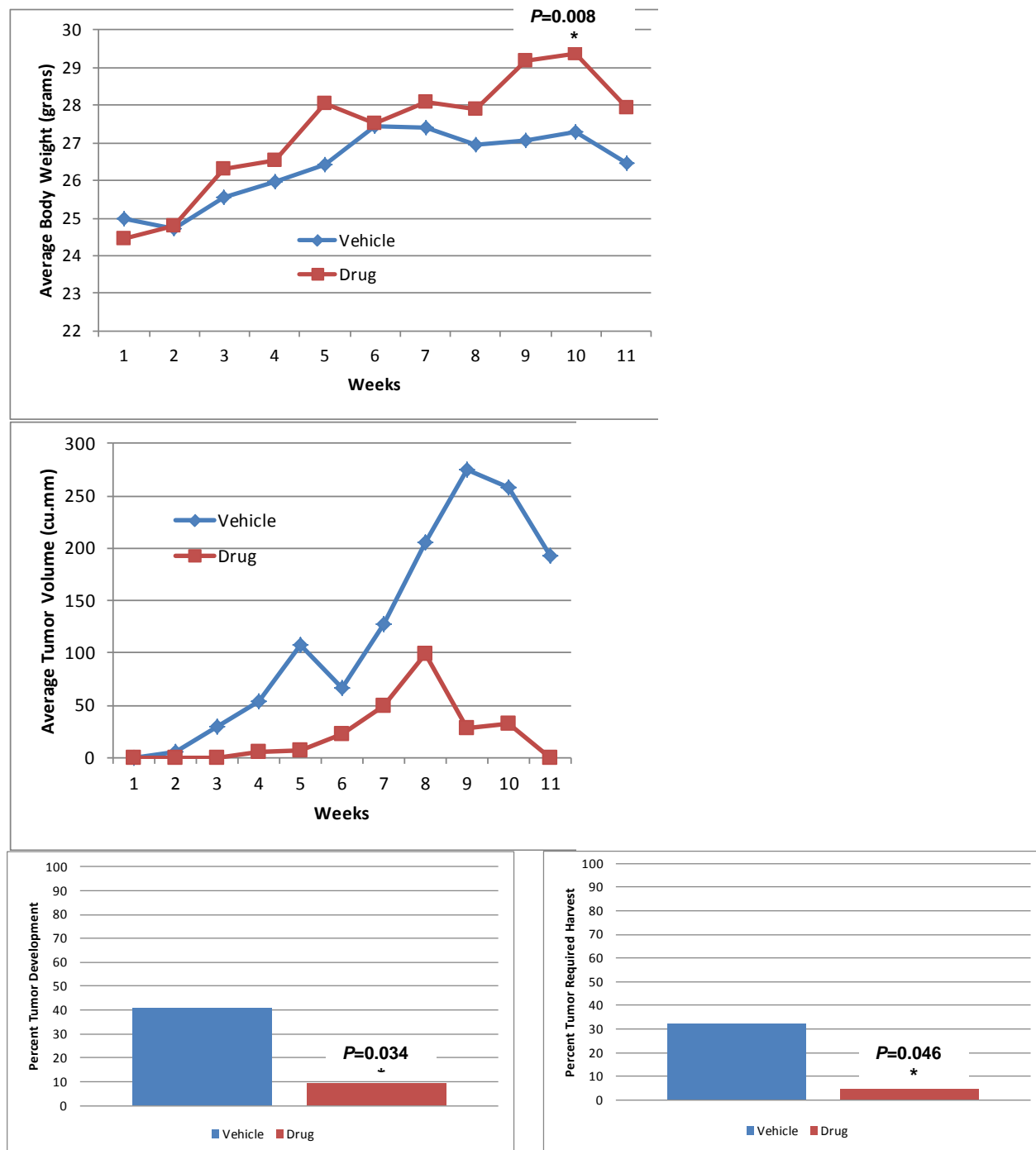


Figure 2. Efficacy of GWARDJ10-001 against LNCaP and C4-2 human prostate cancer xenografts. 1×10^6 LNCaP (A) or C4-2 (B) human prostate cancer cells were injected in a 50% matrigel DMEM preparation into the left inguinal fat pad of 5-6 week old immuno-compromised athymic nude mice. For the castrate-resistant C4-2 (CR C4-2) model, mice were castrated one day prior to xenograft injection. Starting at three weeks post xenograft, mice were treated orally once daily five days per week with GWARDJ10 at the maximum tolerated dose (MTD) of 50 mg/kg or a 60% DMSO saline (vehicle control) solution using a 5 ml/kg body weight dosing volume (N=22 per group between two studies). Mice were weighed at each treatment and palpated for tumor development and size at least once weekly to measure tumor growth. Efficacy was determined based on body weight, and tumor development, growth and euthanasia required due to tumor burden over time. Percent Tumor Development: % of mice that developed tumors; Percent Tumor Required Harvest: % of mice requiring harvest due to tumor size/ decrease in body weight or condition prior to timed end of study harvest. *Statistical Analyses:* Body weight data was analyzed by a repeated measures model with a first-order autoregressive moving-average covariance structure to model the correlation of the multiple body weights measurements over time. Comparison of tumor volume between treatment groups was performed using a non-parametric Wilcoxon rank sum test. Comparison of percent tumor development and percent tumor required harvest between treatment groups were performed using Fisher's exact test. Significant P values are shown *.

A study of chemopreventive efficacy against the TRAMPxFVB transgenic model of PCa was completed as proposed. Mice were randomized to Vehicle Control (60% DMSO saline) or GWARJD10-001 at 50 mg/kg daily oral x 5 treatment every week and followed for tumor development and survival, where survival is defined by the requirement to euthanize a mouse due to tumor burden. As shown in Figure 3, no survival benefit was observed for drug-treated mice compared to vehicle control. A subset of mice was followed by longitudinal MRI imaging for prostate tumor growth. As shown in Figure 3, drug did not slow the growth of tumors. Thus, this regimen of GWARJD10-001 did not show chemopreventive efficacy against the TRAMPxFVB mouse model of prostate cancer in this analysis.

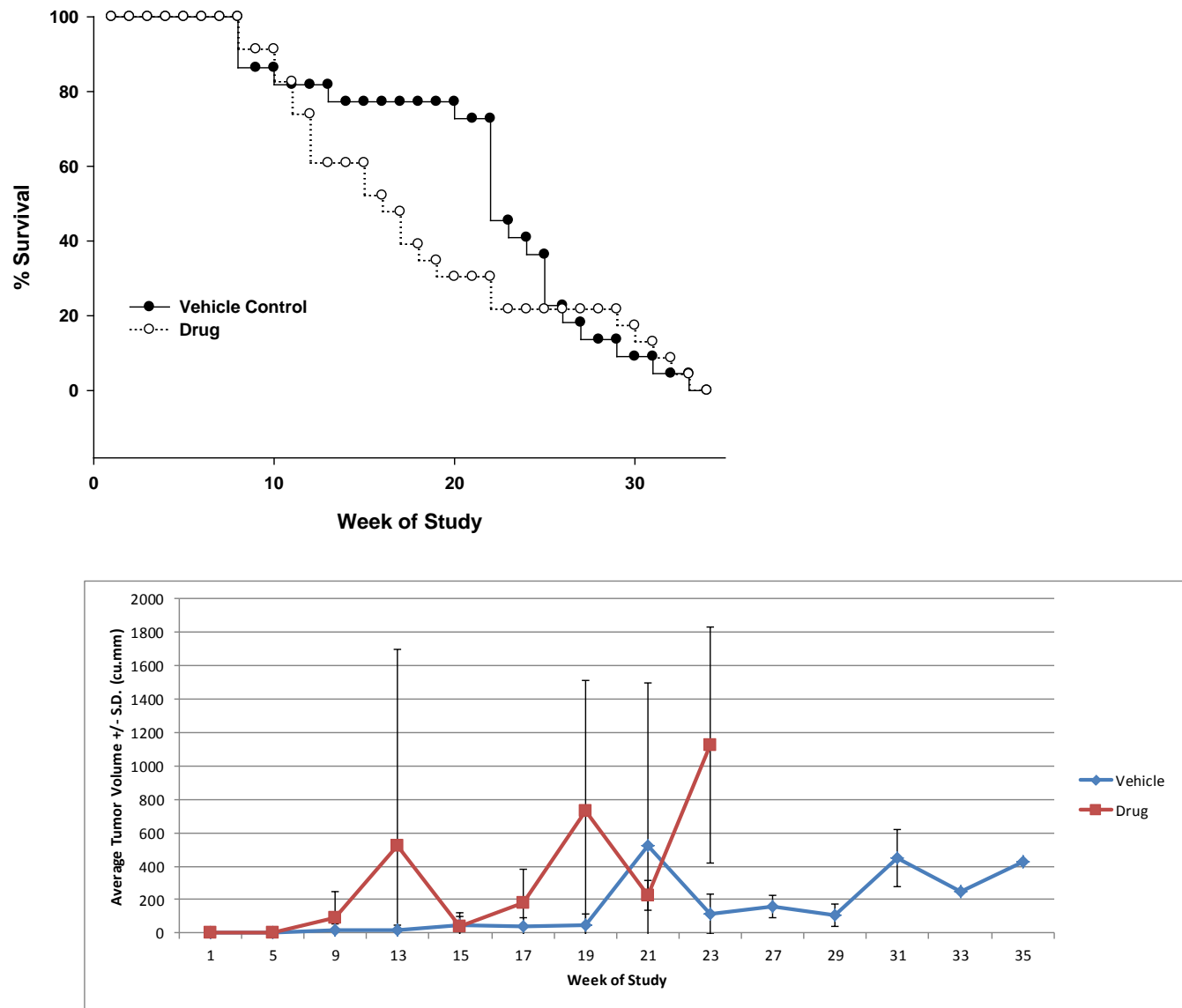


Figure 3. Efficacy of GWARJD10-001 against TRAMPxFVB transgenic mouse model of prostate cancer. TRAMPxFVB[F1] male mice of age 6 to 8 weeks were randomized to treatment with drug at 50 mg/kg (n=23) or vehicle control (n=22) oral daily x 5 every week until euthanization due to tumor burden. Survival (top panel) is defined by the requirement to sacrifice a mouse due to tumor burden. A subset of vehicle-treated (N=6) and drug-treated (N=7) mice were followed by longitudinal MRI imaging for tumor growth (bottom panel).

KEY RESEARCH ACCOMPLISHMENTS

- Identified relatively more important sequences in SSAT promoter for androgen induction of SSAT
- Successfully optimized and used the AR-JunD GL-reconstitution high throughput screen to identify 13 AR-JunD inhibitors from a Life Chemicals (LC) library
- Established that 12 of the 13 AR-JunD inhibitors had the desired non-antiandrogenic property
- Identified 2 non-antiandrogenic compounds with significant activity against ROS production and PCa cell growth *in vitro*
- Successfully optimized and used a novel quantitative protein-protein interaction ICC method to verify the increase in AR-JunD interaction following androgen stimulation and further confirm the ability of the lead compound to specifically inhibit this interaction
- Additionally established that the lead compound blocks induction of SSAT gene expression in the AR-JunD ROS-generating pathway, and mechanism of growth inhibition in PCa cells likely involves reduction of cell cycle regulator cyclin D1
- Successfully developed and used a LC-MS method to perform a PK analysis on the lead compound to establish that 1) the compound has oral bioavailability ideal for extending to future clinical application and 2) nM levels are achieved in the plasma following oral administration and drug is fully cleared from plasma within 24 hours
- Established that the lead compound MTD = 50mg/kg in oral once daily x 5 per week regimen and demonstrated efficacy of the lead compound in this regimen against castrate-resistant C4-2 xenograft PCa model, though no efficacy was observed for androgen-dependent LNCaP xenograft or TRAMPxFOB transgenic PCa models
- Overall we established that AR-JunD is a viable new target for PCa and discovered inhibitors of AR-JunD that show promise for development as new therapeutic agents for PCa

REPORTABLE OUTCOMES

- Kegel S, Mehraein-Ghomi F, Reuter Q, Schmidt J, Church D, Hoffmann FM, Basu H, and Wilding G. Targeting a novel pathway for prostate cancer therapy [abstract]. In: Proceedings of the 102nd Annual Meeting of the American Association for Cancer Research; 2011 Apr 2-6; Orlando, Florida. Philadelphia (PA): AACR; 2011. Abstract nr 2583
- Schmidt J, Kegel S, Mehraein-Ghomi F, Saphner E, Reuter Q, Church D, Hoffmann FM, Basu H, and Wilding G. Inhibiting Androgen Receptor and JunD protein interaction to prevent prostate cancer progression [abstract]. In: Proceedings of the 103rd Annual Meeting of the American Association for Cancer Research; 2012 Mar 31 - Apr 4; Chicago, Illinois. Philadelphia (PA): AACR; 2012. Abstract nr 2844
- Mehraein-Ghomi F, Kegel SJ, Church DR, Schmidt JS, Reuter QR, Saphner EL, Basu HS, Wilding G. Targeting androgen receptor and JunD interaction for prevention of prostate cancer progression. *Prostate*. 2014;74(7):792-803. PMID: in process.
- Plasmids developed: Four different mutant SSAT promoter-luciferase reporter constructs with various degrees of truncation of the SSAT promoter connected to a luciferase reporter in a pGL4 vector; Ap1/JunD sequence point-mutated SSAT promoter-luciferase reporter constructs
- SBIR Phase I grant proposal submitted December 2010, resubmitted December 2011, funded September 2013, 1R43CA162734-01A1 "Androgen Receptor-JunD Complex Inhibitors to Prevent Prostate Cancer Progression" (Colby Pharmaceutical Company PI Hirak Basu, UW-Madison PI George Wilding)

CONCLUSION

We have established that point mutations in the AP1/JunD consensus sequence (TGA/TCA) do not affect the binding of JunD to the SSAT promoter under androgen stimulation. However, removing

bigger sequences in the SSAT promoter did significantly affect the androgen-stimulated SSAT promoter activity, and indicated a 3-dimensional structure where certain sequences important for androgen-induced SSAT activation may be hidden.

We have successfully identified drug-like small molecules that specifically inhibit the AR-JunD interaction downstream of androgen activation of the AR, and validated significant activity against androgen-induced ROS production and androgen-dependent and -independent growth in human PCa cells by two of these AR-JunD inhibitors. We also showed that the lead compound has significant activity against co-translocation of AR and JunD to the nucleus and the interaction of AR and JunD in the PCa cells, as well as ability to block the induction of SSAT gene expression in the AR-JunD ROS-generating pathway in PCa cells. Thus, this research has established that compounds that specifically inhibit the interaction of AR and JunD can block the AR-JunD ROS-generating pathway and inhibit growth of PCa cells.

We have further established that the lead AR-JunD inhibitor is orally bioavailable in mice, albeit at nM level in plasma, which is significantly lower than *in vitro* IC₅₀ / EC₅₀ μ M levels. Despite low bioavailability, the compound showed efficacy against the C4-2 castrate-resistant xenograft PCa model. However, lack of efficacy against the androgen-dependent LNCaP and TRAMPxFOB PCa models suggests its application more for the treatment of CRPCa than as a chemopreventive or early-stage PCa therapeutic agent. Further pre-clinical animal studies are needed to support translation of the lead compound to clinical testing. Since percent tumor development was lower than anticipated for C4-2 Control mice in this study, we are performing another study, supported by other funds, to verify the efficacy against this CRPCa model. Additionally, further analyses on tumors collected from the *in vivo* studies are planned to determine the tissue PK and effect of drug on the target AR-JunD interaction and markers of the AR-JunD ROS-generating pathway, and to examine the difference in action against castrate-resistant PCa compared to androgen-dependent PCa.

Overall the data supports AR-JunD inhibitors as a new class of agents for further research and development as new therapies for early-stage recurrent prostate cancer patients, who have no approved therapy and represent a long unmet medical need. A provisional patent is in place for the compounds discovered by this research and we are in discussion with a Pharmaceutical company to pursue full patent and development of compounds. Further research may include exploration of chemical modifications of the lead compound to improve bioavailability and additional studies in other preclinical models of PCa.

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PERSONNEL

Staff who received pay from this research effort include:

Basu, Hirak S.

Church, Dawn R.

Hoffmann, F Michael

Kim, Kyungmann

Mehraein-Ghomi, Farideh

Weichmann, Ashley M.

Wilding, George

Zhong, Weixiong

APPENDICES

Appendix 1: Mehraein-Ghomi, *et al* publication in *The Prostate*

APPENDIX 1

Publication in *The Prostate*

Targeting Androgen Receptor and JunD Interaction for Prevention of Prostate Cancer Progression

Farideh Mehraein-Ghomi,¹ Stacy J. Kegel,¹ Dawn R. Church,¹ Joseph S. Schmidt,¹ Quentin R. Reuter,¹ Elizabeth L. Saphner,¹ Hirak S. Basu,¹ and George Wilding^{1,2*}

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BACKGROUND. Multiple studies show that reactive oxygen species (ROS) play a major role in prostate cancer (PCa) development and progression. Previously, we reported an induction of Spermidine/Spermine N¹-Acetyl Transferase (SSAT) by androgen-activated androgen receptor (AR)-JunD protein complex that leads to over-production of ROS in PCa cells. In our current research, we identify small molecules that specifically block AR-JunD in this ROS-generating metabolic pathway.

METHODS. A high throughput assay based on *Gaussia* Luciferase reconstitution was used to identify inhibitors of the AR-JunD interaction. Selected hits were further screened using a fluorescence polarization competitor assay to eliminate those that bind to the AR Ligand Binding Domain (LBD), in order to identify molecules that specifically target events downstream to androgen activation of AR. Eleven molecules were selected for studies on their efficacy against ROS generation and growth of cultured human PCa cells by DCFH dye-oxidation assay and DNA fluorescence assay, respectively. In situ Proximity Ligation Assay (PLA), SSAT promoter-luciferase reporter assay, and western blotting of apoptosis and cell cycle markers were used to study mechanism of action of the lead compound.

RESULTS. Selected lead compound GWARD10 with EC₅₀ 10 μM against ROS production was shown to block AR-JunD interaction in situ as well as block androgen-induced SSAT gene expression at IC₅₀ 5 μM. This compound had no effect on apoptosis markers, but reduced cyclin D1 protein level.

CONCLUSIONS. Inhibitor of AR-JunD interaction, GWARD10 shows promise for prevention of progression of PCa at an early stage of the disease by blocking growth and ROS production. *Prostate* 74:792–803, 2014. © 2014 Wiley Periodicals, Inc.

KEY WORDS: AR-JunD protein-protein interaction; small molecule inhibitors; cellular reactive oxygen species; cyclin D1

INTRODUCTION

Advanced hormone refractory metastatic prostate cancer (PCa) is the second leading cause of cancer deaths of US men [1]. PCa recurs in approximately 30% of patients after their first-line of therapy of either radical prostatectomy or ionizing radiation [2]. Although Androgen-Deprivation Therapy (ADT) initially causes the regression of the early stage recurrent PCa, unfortunately over 80% of the patients fail ADT and progress to androgen-independent castration-resistant tumor (CRPCa) [2]. Therefore, identification of an effective agent to prevent PCa progression at an early stage of recurrence is a major focus for PCa research.

Grant sponsor: Department of Defense; Grant number: W81XWH-10-1-0169; Grant sponsor: National Institutes of Health grant; Grant number: P30 CA014520 (UWCCC CCSG); Grant sponsor: Prostate Cancer Foundation.

Mehraein-Ghomi and Kegel contributed equally to this work.

Conflict of interest: Farideh Mehraein-Ghomi, Dawn R. Church, Hirak S. Basu, and George Wilding have potential conflict of interest.

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Cellular Reactive Oxygen Species (ROS), such as hydroxyl radical, superoxide, hydrogen peroxide, and nitric oxide, are naturally occurring carcinogens in prostate epithelial cells [3]. When cellular ROS production overwhelms detoxification capacity of cells, oxidative stress occurs [4]. It has been established that oxidative stress causes DNA, RNA, and phospholipid damages [5]. ROS also act as a signal for promoting cell proliferation that can contribute to cancer development and progression, including prostate cancer [3,6,7]. Data published from our and other laboratories have shown that androgen signaling is a major source of ROS generation in prostatic epithelial cells [8–11]. We have further shown that one pathway of androgen-induced oxidative stress generation involves activation of AP-1 transcription factor JunD [12,13] followed by androgen receptor (AR)-JunD association [14] that induces an enzyme Spermidine/Spermine N¹-Acetyl Transferase (SSAT). Induction of SSAT initiates a major polyamine oxidation pathway that generates excess hydrogen peroxide (H₂O₂) production in polyamine-rich prostatic epithelial cells [15]. We established that JunD is required for androgen-induced SSAT gene expression and ROS generation [14]. We hypothesize that specific inhibitors of AR-JunD interaction that act downstream of AR activation will specifically inhibit androgen induced SSAT gene expression and consequently prevent the excess ROS production, and should therefore prevent the initiation and progression of PCa.

In our previously published study we used a *Gaussia* luciferase enzyme reconstitution assay to demonstrate a direct interaction of activated AR with JunD [14]. Here, we performed a high throughput screen of NCI Diversity Set [16] and Life Chemicals [17] small molecule libraries to identify potential candidates that may inhibit this interaction without anti-androgenic activity due to binding to the AR-ligand binding domain (LBD). Selected compounds have been further characterized for ROS and cell growth inhibitory effects. One of the lead compounds, GWARD10, significantly reduced androgen-induced ROS production in LNCaP cells, as well as proliferation of androgen-dependent LNCaP and castrate-resistant C4-2 cells. GWARD10 was studied further to confirm its proposed mechanism of action in blocking PCa progression.

Studies on the mechanism of action of GWARD10 showed that this compound significantly reduces the interaction between AR and JunD and also reduces the transcriptional activity of SSAT promoter in LNCaP human PCa cells cultured in the presence of androgen. Further studies showed that this compound significantly reduces cyclin D1 expression in the presence of androgen in both LNCaP and C4-2 cells. No significant

effect on apoptosis markers (e.g., cleaved PARP) at this concentration of the compound was observed in either of the two cell lines.

MATERIALS AND METHODS

Cell Culture

Androgen-dependent LNCaP human prostate carcinoma cells were obtained from the American Type Culture Collection. Castrate-resistant LNCaP C4-2 cells [18] were a kind gift from Ajit Verma (Department of Human Oncology, UW-Madison), with permission from George Thalmann (Department of Urology, Inselspital, Bern, Switzerland). LNCaP cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% fetal bovine serum (FBS) and grown in the same medium (F5 medium) for androgen-dependent growth studies. For studies on androgen-induced ROS, LNCaP cells were grown in DMEM supplemented with 1% FBS and 4% charcoal stripped serum (F1C4 medium) as described before [8]. This combination of stripped and non-stripped serum was previously shown to sufficiently deplete androgen content while limiting the adverse growth effects not related to hormone depletion that occur with the use of 5% stripped serum [8]. LNCaP C4-2 cells were maintained and grown for androgen-independent growth studies in the F1C4 medium.

Hep3B human hepatoma cells with no endogenous AR were obtained from the Small Molecule Screening and Synthesis Facility at the University of Wisconsin Carbone Cancer Center (UWCCC SMSSF) and maintained in RPMI 1640 supplemented with 10% FBS and antibiotics, as explained previously [14]. Synthetic androgen R1881 (methyltrienolone, NEN) was used as an androgen analog in cell culture studies at 1–2 nmol/l for maximal induction of JunD and ROS as described before [12]. For Hep3B transfected cells, 2 nmol/l R1881 was used to induce the activity of AR after transfection.

For direct cDNA synthesis from cells for qRT-PCR, 1.7×10^5 LNCaP cells were cultured in each well of a 12 well plate in F1C4 medium, treated with or without 2 nM R1881 and 5 μ M GWARD10, and incubated for 24, 48, and 72 hr. After incubation, cells from each time point were lysed by a lysis solution provided in a kit by Life Technologies™ following the manufacturer instructions (see below).

Antibodies Used for Western Blot and In Situ Proximity Ligation Assay (PLA)

Mouse monoclonal antibody for cyclin D1 (Santa Cruz, sc-8396), mouse monoclonal antibody for

cleaved PARP (Cell Signaling #9546), mouse monoclonal antibody for Androgen Receptor (Santa Cruz, sc-7305), and rabbit polyclonal antibody for JunD (Santa Cruz, sc-74) were used. β -actin mouse monoclonal antibody (AC-15) for loading and normalization control was obtained from Sigma-Aldrich.

Transfection of Constructs cmv-Gluc1-AR and cmv-JunD-Gluc2 Into Hep3B Cells and Generation of Large Scale Cell Lysate for HighThroughput Screening

Transfection of cells was done using Lipofectamine 2000 reagent (Invitrogen) per manufacturer's instructions as explained previously [14]. Briefly, Hep3B cells were seeded and 1 day later co-transfected with 3 μ g each of cmv-Gluc1-AR and cmv-JunD-Gluc2 constructs. Transfections with cmv-Gluc1-AR or cmv-JunD-Gluc2 alone were used as negative controls, and cmv-Gluc1-smad3 co-transfected with cmv-PKB-Gluc2 [19] was used as a positive control. Two to 3 hr after transfection, cells were washed and refed with DMEM without serum and either treated with 2 nmol/l R1881 (+R) or left untreated (–R). The cells were incubated after transfection at 37°C under 5% CO₂ for 48 hr, then lysed using a lysis buffer provided in a *Gaussia* luciferase assay kit from New England Biolabs (Ipswich, MA) following the manufacturer's protocol.

Initial HighThroughput Screening (HTS) of NCI Diversity Set Library Containing ~2000 Small Molecules and Life Chemicals Library Containing 25,000 Drug-Like Small Molecules

Five microliter of cell lysate per well was plated in 384 well plates using MicroFlo Select (BioTek Instruments, Inc., Winooski, VT). Two columns in each plate were used for negative controls (for DMSO and for lysates from cells that were transfected with both constructs but were not treated with R1881). One hundred nanoliters of 1 mmol/l of each potential inhibitor (final concentration of 20 μ mol/l for each compound) and control DMSO from the NCI Diversity Set and Life Chemicals Library were added to each well using a robotic pin-based liquid handler (Biomek FX, Beckman Coulter, Inc.) following a procedure published by the UWCCC SMSSF [20]. The plates were sealed by MicroSeal B Film (BioRad) and incubated overnight at 37°C in dark. Ten microliter of Renilla Luciferase substrate (Promega) was added to each well, and the bioluminescence activity of *Gaussia* luciferase in lysates was measured using a Synergy 4 plate scanner (BioTek, Winooski, VT). Compounds that blocked greater than 30% of the luciferase activity

were considered as "hits" in this and all subsequent screening assays. Z' factor values of above 0.5 are generally accepted as significant for identification of "hits" in a HTS assay [21]. For all screens, our Z'-factor was above 0.56.

Secondary Screen of the Identified Hits of the Initial HTS

For eliminating false positives, including non-specific inhibitors and toxins, 5 μ l lysate/well from cells that were co-transfected with vectors containing cmv-Gluc1/cmv-Gluc2 (negative control) or with cmv-smad3-Gluc1/cmv-Gluc2-PKB (positive control) [19] were plated in a 384 well plate and 0.1 μ l of each identified inhibitor from 1 mmol/l stock (final concentration of 20 μ mol/l) was added to each well. Plates were sealed as before and incubated for an overnight at 37°C in dark, then the bioluminescence activity was measured as described earlier. Only the "hits" that inhibit *Gaussia* Luciferase reconstitution in an AR-JunD system, but fail to inhibit GL-reconstitution in the secondary screen with the positive control were considered as "true" inhibitors of the AR-JunD complex (Fig. 1, "Confirmed Hits").

Fluorescence Polarization AR Ligand Binding Competition Assay

Since inhibition of AR activity and its interaction with JunD might have been due to the competition of these inhibitors with androgen for binding to the Ligand Binding Domain of AR (AR-LBD) and thus blocking androgen activation of AR, we used a commercially available assay to determine the anti-androgenic AR-LBD binding activity of these inhibitors. Selected hits from the dual HTS of the NCI Diversity Set and Life Chemical libraries were tested by PolarScreen™ Androgen Receptor Competitor Assay kit (Invitrogen) to determine their abilities for binding to the AR-LBD. Fluorescence polarization assay with graded concentrations of the compounds of interest was performed according to the manufacturer's supplied protocol, in comparison to clinical antiandrogen Casodex® (bicalutamide).

Growth and ROS Assays

Cells collected for experiments were counted and seeded in 96-well tissue culture plates at a density of 4,000 cells per well in 200 μ l medium described above 1 day prior to treatment with compounds in varying doses. Androgen treatments for ROS studies were done on the same day. Treatments were carried out over 96 hr at 37°C under 5% CO₂. Measurements of

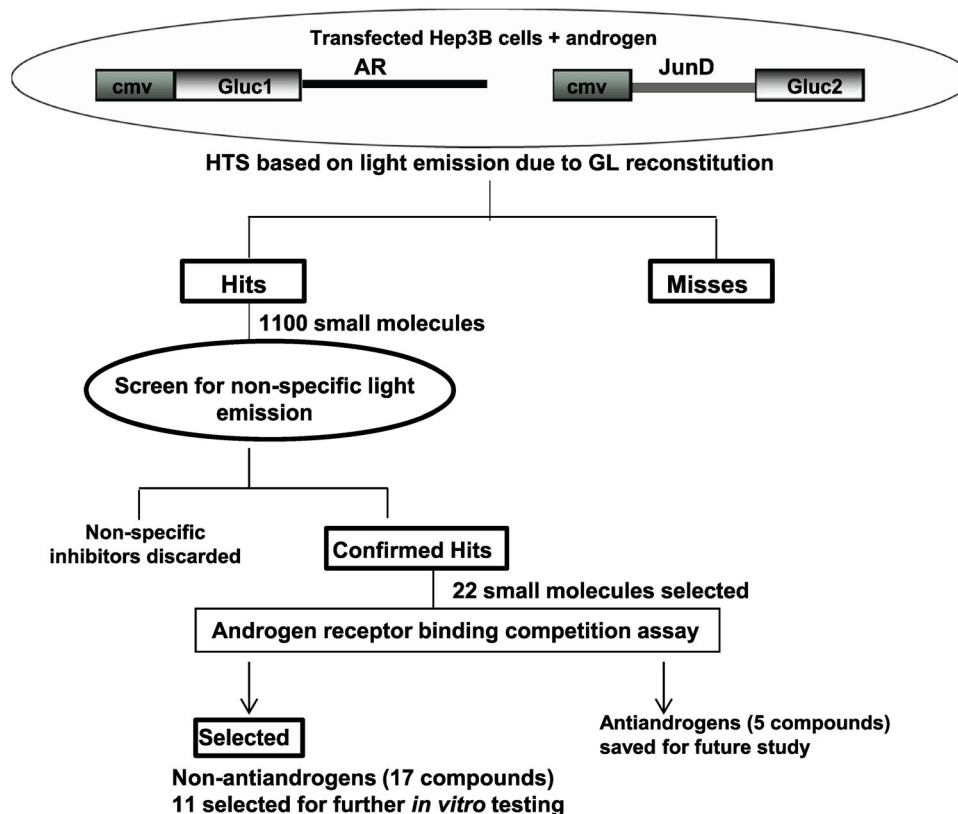


Fig. 1. Schematic presentation of high throughput screen and selection of small molecule inhibitors of AR-JunD interaction. NCI Diversity Set library of ~2,000 small molecules and a Life Chemical Library of 25,000 small molecules were assayed in a high throughput screen (HTS) based on a Gaussia Luciferase (GL) reconstitution due to AR and JunD interaction (see Methods). Z' factor for all screens was >0.56 . Eleven hundred small molecules that showed $>30\%$ inhibition of GL in the first screen were further screened with *cmv-smad3-Gluc1/cmv-Gluc2-PKB* control to eliminate false positives. Twenty-two confirmed hits were considered as compounds of interest for further testing and screened for their ability to compete with androgen for binding to androgen receptor ligand-binding domain (AR-LBD; see Fig. 2), to eliminate antiandrogens since our focus is on inhibiting AR post androgen activation. Eleven of the non-antiandrogens were selected for further *in vitro* testing based on their structure and/or greater than 50% block of luciferase activity in the first HTS.

ROS were used as an indicator of redox status and DNA levels were measured as an indicator of growth. The 96-well culture plates were assayed for estimation of ROS levels in intact cells using the fluorescent dye 2', 7'-dichlorofluorescein di-acetate (DCF; Molecular Probes, Inc.) following a published procedure [22]. In brief, cell cultures were washed with 200 μ l Krebs's Ringer buffer prewarmed to 37°C, incubated under 5% CO₂ at 37°C in 100 μ l Krebs's Ringer buffer containing 10 μ g/ml DCF dye for 45 min. Each 96-well culture plate was scanned on a Synergy 4 plate scanner (Biotek) using the 485/530 nm filter excitation and emission set and then frozen at -70°C for the subsequent analysis of DNA content. For DNA analysis, each culture plate frozen to -70°C was equilibrated to room temperature protected from light. Hoechst dye was then added to each well at a final concentration of 6.7 μ g/ml in 200 μ l of high salt TNE buffer (10 mmol/l Tris, 1 mmol/L EDTA, 2 mol/L NaCl [pH 7.4]). After further incubation at room temperature for over 2 hr

under protection from light, culture plates were scanned on a Synergy 4 plate scanner using the 360/460 nm filter excitation and emission set. The DCF fluorescence units were normalized to the Hoechst-DNA fluorescence units for each well and used as a measure of the level of ROS being generated. The DNA fluorescence units were also used as a measure of cell growth.

Quantitative In Situ Proximity Ligation Assay (PLA)

For quantifying the level of inhibition of AR-JunD complex formation by the lead compound GWARDJ10, we performed a quantitative in situ Proximity Ligation Assay (PLA) [23] in intact cells using a kit from OLINK Bioscience (Uppsala, Sweden). LNCaP cells (2×10^4 cells/well) were seeded in eight-well polylysine coated chamber slides in F1C4 media [8] and treated with 2 nmol/l R1881 (positive control) or without R1881

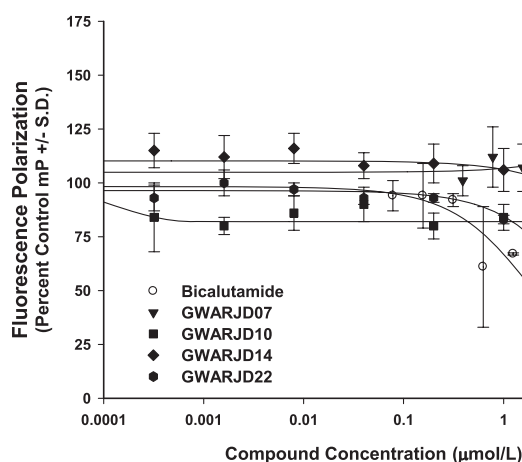


Fig. 2. Screening of compounds for anti-androgenic properties. The PolarScreen™ Androgen Receptor Competitor Assay was used to determine if the confirmed hits have anti-androgenic properties. Bicalutamide (Casodex®), a clinical antiandrogen, was used as a control. A classic dose response of bicalutamide competition with fluorescence tagged androgen (fluoromone) for binding to the ligand binding domain (LBD) of AR was observed. In contrast, the GWARJD inhibitors numbers 7, 10, and 14, which are the focus of this paper, did not compete with androgen for binding to LBD of AR in the same dose range. All 22 selected confirmed hits were tested in this manner, and based on this assay 17 were classified as non-anti-androgens while 5 were classified as antiandrogens, as represented by GWARJD22 showing a curve similar to bicalutamide. N = 3 per data point, nonlinear regression fit curves, representative from two assays performed for each compound.

(negative control) in control channels, or treated with or without 2 nmol/l R881 with 5 μmol/l of GWARJD10 inhibitor. In brief, after 72 hr incubation at 37°C under 5% CO₂, cells were washed and then fixed in 4% Paraformaldehyde in PBS for 10 min at room temperature. Cells were then permeabilized with 0.5% Triton-X100 in PBS for 10 min at room temperature. Subsequently cells were washed with 0.05% Tween 20 in PBS (PBS-T). The instructions provided by manufacturer were followed for PLA assay. Briefly, after the cells were permeabilized and washed with PBS-T, one drop of Duolink II Blocking Solution (1×) was added to each well after complete removal of wash buffer, slides were incubated for 1 hr at 37°C in a 5% CO₂ incubator. After 1 hr, the blocking solution was completely removed and 1/100 dilution of rabbit polyclonal antibody for JunD and 1/100 dilution of mouse monoclonal antibody for AR were added to each well and in one well only antibody diluent was added as a negative control. Slides were incubated for 1 hr at 37°C under 5% CO₂ and then washed with supplied wash buffer. Forty microliter of two PLA probes (Duolink II anti-Mouse MINUS and Duolink II anti-Rabbit PLUS) at 1:5 in antibody diluent was added to each well. After 1 hr incubation at 37°C under 5% CO₂, slides were washed

and 40 μl Duolink II Ligation stock (×5) was added to each well and incubated for 30 min at 37°C under 5% CO₂. After incubation, slides were washed and 40 μl of diluted Duolink II Amplification Mix was added to each well and incubated for 100 min at 37°C under 5% CO₂. Finally, slides were washed and mounted using one drop of solution containing DAPI. A Nikon TI-U inverted fluorescence microscope with excitation/emission 594/624 filters was used to acquire images of PLA-processed slides at ×20 magnification. The red fluorescent spots were counted and normalized against nuclei blue DAPI for a quantitative measure of AR-JunD interaction.

SSAT Full Length Promoter-Luciferase (*ssatP-luc*) Reporter Assay

LNCaP (1 × 10⁵) cells were seeded in each well of 12-well polylysine coated plates. The day after seeding, cells were co-transfected with 2 μg of the previously published [14] *ssatP-luc* vector and 0.1 μg of cmv-betagalactosidase (internal control for transfection) (Promega) using Lipofectamine 2000 (Invitrogen) and following the manufacturer's instructions. After transfection, cells were treated with or without 2 nmol/LR1881 (± R) with 1 or 5 μmol/l of GWARJD10 or zero dose vehicle control.

Luciferase assay was performed using a kit from Promega as instructed by the company and described previously [14], for quantitation of androgen-induced SSAT promoter activity.

PSA cDNA Synthesis and Quantitative Real Time PCR (qrtPCR)

Cells-to-cDNA™ II, reverse transcription without RNA isolation kit from Ambion® by Life Technologies™ (AM 1723) was used to synthesize cDNA directly from cells following the instructions provided by the manufacturer. Briefly, 100 μl of lysis solution was added to cells in each well of a 12 well plate, and the lysed cells were incubated at 75°C for 10 min. The genomic DNA was lysed by adding 2 μl of DNaseI per 100 μl lysed cells and incubated for 15 min at 37°C. The DNaseI was inactivated by heating the solution to 75°C and the reverse transcription of released RNA was achieved by incubating the lysates at 42°C for 1 hr using reverse transcription mix provided in the kit. The reverse transcription was stopped by heating the samples to 95°C for 10 min. The cDNAs were subjected to real time PCR using IQ™ Syber® Green supermix (Bio-Rad). Each reaction was normalized by coamplification of 18srRNA. Triplicates of samples were run on a Bio-Rad CFX-96 real-time cyler. The sequences of

primers used for amplification of PSA and 18srRNA cDNAs were as follows:

PSA: forward: 5'-GACCACCTGCTACGCCTCA and reverse: 5'-GGAGGTCCACACTGAAGTTTC

18srRNA: forward 5'-CGCCGCTAGAGGTGAAATCT and reverse 5'-CGAACCTCCGACTTTCGTT.

Western Blot

Cell lysates were prepared using modified Radio-immunoprecipitation (RIPA) buffer (50 mmol/l Tris-HCl, PH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mmol/l NaCl and 1 mmol/l EDTA) containing a tablet of complete protease inhibitors from Roche (Indianapolis, IN). Total protein from each sample was separated on a 4–12% Bis-Tris gel NuPAGE Novex from Life Technologies. The gel was transferred onto a PVDF membrane, blocked and then incubated with antibody as explained before [14]. The membrane was developed with Enhanced Chemiluminescent (ECL) substrate from Thermo Scientific after incubation with the appropriate HRP-conjugated secondary antibodies.

Statistical Analyses

An unpaired two-tailed heteroscedastic Student's *t*-test with a confidence level of 0.05 was performed for data comparisons and significance determination.

RESULTS

Identification of Specific Inhibitors of AR-JunD Interaction

For identification of specific inhibitors of the AR-JunD interaction, a NCI Diversity Set library containing 2,000 small molecules and Life Chemicals Library containing 25,000 drug-like small molecules were screened using *Gaussia* luciferase reconstitution assay as explained in the Materials and Methods (see Methods). The screening scheme is shown (Fig. 1). The assay used conditions where both AR and JunD proteins were overexpressed in a non-prostate cancer cell line (Hep3B cells) without AR background. This cell line was selected to avoid the competition from binding of the endogenous AR. Eleven hundred molecules were identified as inhibitors in this first screen, based on greater than 30% block of luciferase activity. The second screen was performed using the cell lysates from the positive smad3/PKB control [19] (see Methods). Molecules that inhibited the interaction of smad3/PKB were considered to be non-specific inhibitors and were discarded. Only the "confirmed hits" from the second screen were considered as specific inhibitors of AR-JunD interaction. Twenty-two

such molecules were considered as compounds of interest and selected for further testing and analysis.

Lack of AR-LBD Binding Activity of Specific Inhibitors of AR-JunD Interaction

Using the PolarScreen™ Androgen Receptor Competitor Assay kit from Invitrogen, all 22 selected confirmed specific inhibitors of AR-JunD interaction were tested for their ability to bind to the AR Ligand Binding Domain (AR-LBD). Data for select compounds are shown (Fig. 2). Antiandrogens, such as the positive control bicalutamide and compound GWAJRD22 shown (Fig. 2), exhibited a dose dependent decrease in polarization value. As our goal is to specifically target and block steps *downstream* to AR activation by androgen, the five compounds found to be antiandrogens by this assay were set aside and not studied further at this time. Seventeen compounds that did not show a decrease in polarization value, which indicates their inability to compete with androgen for binding to AR-LBD, were considered for further studies.

AR-JunD Inhibitors Prevent Cell Growth in Both LNCaP and Its Castrate-Resistant Variant C4-2 Cells in Culture

Of the 17 specific inhibitors of AR-JunD interaction selected from the dual HTS and AR-LBD assay data described above, 11 compounds were selected for further studies based on their structures that allow for ease in chemical synthesis and/or greater than 50% block of luciferase activity in the first HTS. These 11 compounds were tested in cell culture for efficacy against the androgen-induced ROS production in which AR-JunD interaction plays a key role [14], as well as efficacy against androgen-dependent and -independent PCa cell growth.

Data obtained from the ROS and cell growth studies for the three most effective compounds, GWAJRD07 (NSC693573), GWAJRD10 (F1174-3266), and GWAJRD14 (F3382-1718), are shown (Fig. 3), with their structures shown (Fig. 3a). Results from GWAJRD07 (CPC507) showed that at 0.8 μ mol/l, it causes a marked inhibition of androgen-induced ROS production in androgen-dependent LNCaP cells (Fig. 3b) and inhibits growth by 50% at less than 500 nmol/l ($IC_{50} < 500$ nmol/l; Fig. 3c). In castrate-resistant C4-2 cells, GWAJRD07 showed a marked effect at 30 nmol/l on cell growth and its IC_{50} is less than 100 nmol/l (Fig. 3c). This compound, however, has an imidazole ring and thus, has anti-oxidant properties. As chemical anti-oxidants such as alpha-tocopherol failed in PCa therapy/prevention [24], we focused on GWAJRD10 and GWAJRD14 that have no chemical anti-

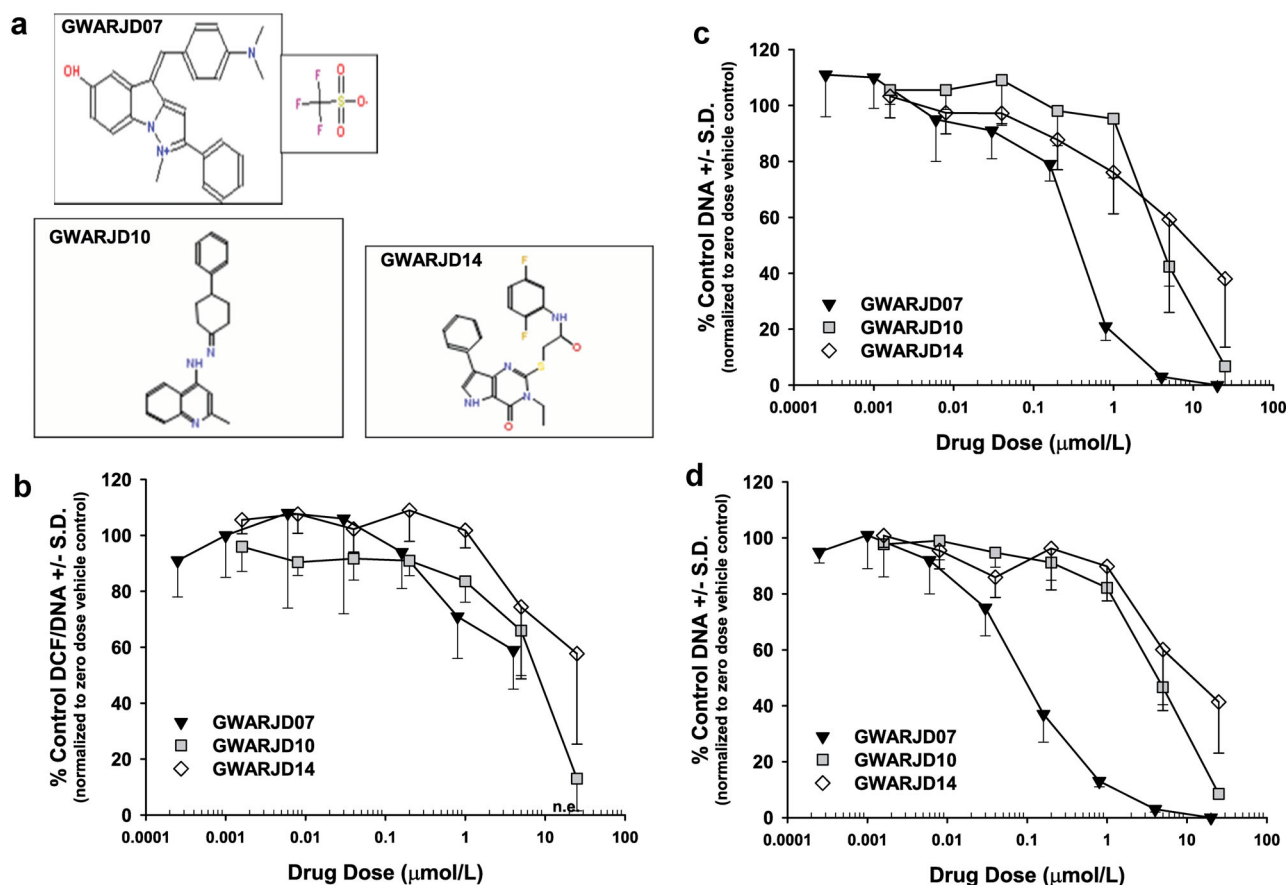


Fig. 3. ROS and growth analysis: compounds (structures shown in **a**) were tested for their ability to block androgen-induced ROS production (**b**) and inhibit androgen-dependent (**c**) and androgen-independent (**d**) prostate cancer cell growth. (**b**) Dose response effect of compounds on ROS in LNCaP treated with 1 nmol/l R1881 and androgen that induces AR-JunD interaction and subsequent ROS over-production. (**c**) Dose responses effect on growth of androgen-dependent LNCaP cells in growth stimulatory androgen complete medium (F5). (**d**) Dose response effect on growth of castrate-resistant variant LNCaP C4-2 cells in androgen-deprived medium (FIC4). All treatments were carried out over 96 hr. Data points are the average from three experiments with $N = 6$ per condition in each experiment ($\#$ s 10 and 14) or $N = 6$ in one experiment ($\#$ 7). n.e., not evaluable due to extremely low DNA values.

oxidant properties. GWARJD10 also showed inhibitory effects on both ROS production and PCa cell growth, with inhibition of androgen-induced ROS production by 50% at less than 10 μmol/l in LNCaP cells ($EC_{50} < 10$ μmol/l; Fig. 3b) and growth inhibition in both LNCaP and C4-2 cells by 50% at less than 5 μmol/l ($IC_{50} < 5$ μmol/l; Fig. 3c and d), respectively. GWARJD14 was found to inhibit androgen-induced ROS production by 50% at approximately 25 μM ($EC_{50} \sim 25$ μmol/l; Fig. 3b), and inhibit both androgen-dependent LNCaP and castrate-resistant C4-2 cell growth by 50% at less than 10 μmol/l ($IC_{50} < 10$ μmol/l; Fig. 3c and d), respectively. A cell viability assay showed no cytotoxicity at the growth inhibitory IC_{50} concentrations for each of these compounds, and the concentrations at which 50% cytotoxicity was observed for each was well above their IC_{50} concentration (data not shown), thus the observed growth inhibition was likely not due to cytotoxic proper-

ties of the compounds. We selected the more effective compound GWARJD10 for confirming its proposed mechanism of action and thereby, establishing the proof-of-principle.

Inhibition of Interaction of AR-JunD by GWARJD10 in Androgen-Dependent LNCaP Cells

Results from the quantitative in situ Proximity Ligation Assay (PLA; see Methods) demonstrate the interaction of AR-JunD in R1881 treated LNCaP cells (Fig. 4a). In androgen-dependent LNCaP cells, R1881 activates Androgen Receptor (AR) and JunD that initiates their interaction and translocation to the nucleus (Fig. 4a), where they may bind to the promoter of genes that are involved in growth and ROS production. In LNCaP cells that were treated with 2 nmol/l R1881 and 1 or 5 μmol/l GWARJD10, AR and JunD

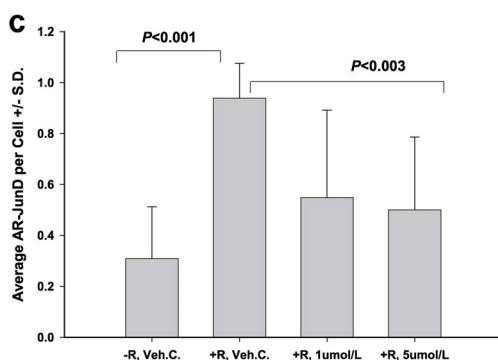
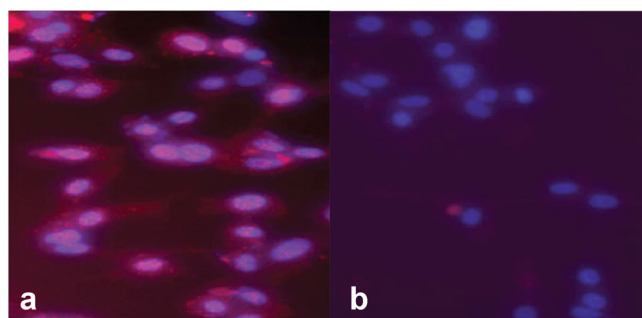


Fig. 4. Inhibition of androgen-induced AR-JunD interaction by GWARJD10 in LNCaP cells. The DuoLink Proximity Ligation Assay (PLA) technique was used to analyze the effect of lead compound GWARJD10 on androgen-induced AR-JunD interaction. Androgen-dependent LNCaP cells were treated for 72 hr either with 2 nmol/l androgen R1881, positive control (a) or with 2 nmol/l R1881 and 5 μ mol/l of GWARJD10 (b). In (a), androgen-induced interactions of AR with JunD are represented by red spots in the cytoplasm and by pink spots in the nuclei which are the merge of red spots with blue DAPI. With the addition of GWARJD10 (b), the interactions of the two proteins were inhibited and few AR-JunD interaction spots were observed (magnification 20 \times). In (c), LNCaP cells treated with (+) or without (–) 2 nmol/l androgen R1881 (R) and either 0.25% DMSO drug vehicle control (Veh.C.) or 1 or 5 μ mol/l GWARJD10 for 72 hr were analyzed by PLA for AR-JunD interaction. Fluorescent spots resulting from AR-JunD interaction (red) were counted and normalized to the number of cells (blue, DAPI) per field using an inverted fluorescent microscope. The threefold induction of AR-JunD interaction by androgen ($P < 0.001$) was significantly reduced by both 1 and 5 μ mol/l GWARJD10 ($P < 0.003$). The level of AR-JunD interaction in +R, 5 μ mol/L GWARJD10 was not significantly different from androgen-deprived control –R,Veh.C. $N = 12$ fields counted per condition across two experiments (6 fields per experiment).

interaction was significantly abrogated ($P < 0.003$; Fig. 4b and c).

GWARJD10 Significantly Reduces Androgen-Induced Transcriptional Activity of *ssat* Promoter

Previously, we showed that JunD binds to the SSAT promoter [14] and also showed that the

expression of SSAT enzyme is ~ 30 -fold higher in LNCaP cells treated with R1881 compared to the control [15]. Because there is no AR Response Element (ARE) in the SSAT Promoter sequence, an increase in the SSAT gene expression is likely due to the complex formation between AR and JunD, where JunD binds to the AP1 consensus sequence in *ssat* Promoter [14]. Here, we show in (Fig. 5) that 5 μ mol/l GWARJD10 causes a significant threefold reduction in the androgen-induced *ssat* Promoter activity ($P = 0.003$).

GWARJD10 Significantly Reduces AR Transcriptional Activity as Measured by PSA Expression in LNCaP Cells Under Low Androgen (FIC4) Condition But Does Not Affect Androgen Induction of AR Transcriptional Activity

As shown in Figure 5b, GWARJD10 significantly reduced the transcriptional activity of AR, as measured by PSA mRNA expression, in low androgen (–R) environment ($P < 0.01$ for all timepoints for drug treated cells compared to control in –R condition). Stimulation with 2 nM androgen R1881 (+R) significantly increased AR transcriptional activity with respect to PSA expression as expected ($P < 0.001$ for all timepoints for androgen treated cells compared to low androgen without drug), but GWARJD10 had no effect on this induction of AR transcriptional activity by androgen, as levels of PSA mRNA were the same in cells treated with androgen without or with drug (+R, –D compared to +R, +D).

GWARJD10 Reduces the Expression of Cyclin D1 in the Presence of 2 nmol/l R1881 But Does Not Reduce the Expression of cPARP in the Presence or Absence of Androgen

LNCaP and C4-2 cells were treated with vehicle control or with 2 nmol/l R1881 with or without 1 or 5 μ mol/l GWARJD10. We observed a significant decrease in cyclin D1 expression in 2 nmol/l R1881 and 5 μ mol/l GWARJD10-treated LNCaP and C4-2 cells after 72 hr compared to controls. Representative western blots are shown in Figure 6, panels c and d, respectively. Quantitation of the bands showed that cyclin D1 level was significantly reduced on average across independent experiments to $65 \pm 6\%$ in LNCaP cells ($P = 0.001$, $N = 3$) and $71 \pm 13\%$ in C4-2 cells ($P = 0.05$, $N = 4$) as compared to control. We, however, did not observe any significant change in cleaved PARP after treatment with 5 μ mol/l of GWARJD10 when compared to the controls (Fig. 6, panels a–d)

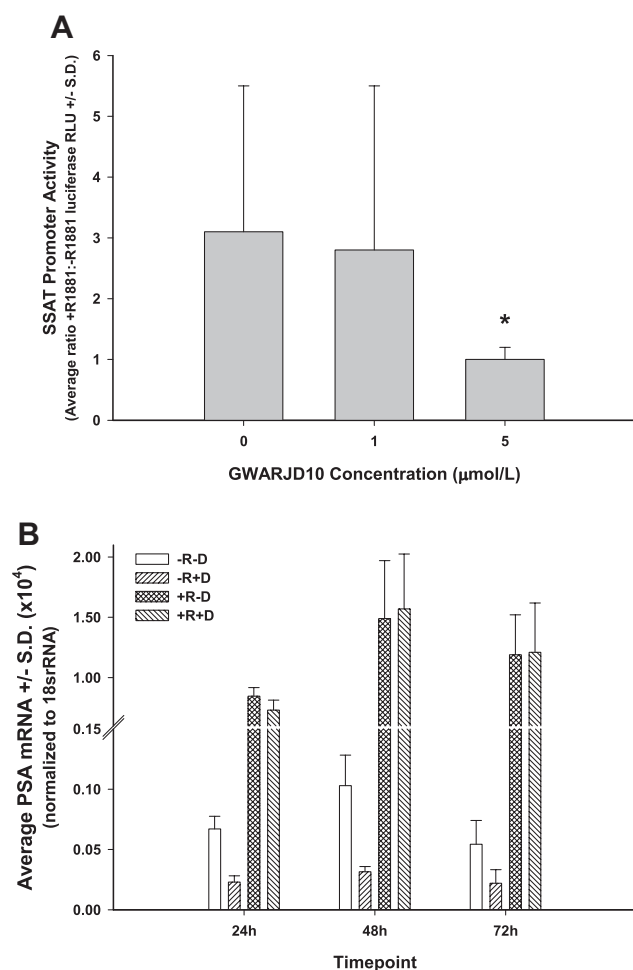


Fig. 5. Effect of GWARJD10 on androgen receptor activity. **(a)** Inhibition of androgen-induced SSAT promoter transcriptional activity by GWARJD10 in LNCaP cells: LNCaP cells were transiently transfected with an SSAT promoter-luciferase reporter vector and treated with 2 nmol/L androgen R1881 or vehicle control with or without 1 or 5 μmol/L GWARJD10. Cell lysates were collected after 72 hr; the relative light units (RLU) of firefly luciferase activity was measured by a luminometer at 480 nm, and the ratio of RLU for androgen treated (+R1881) versus vehicle control (–R1881) cells was determined. Similarly, the ratios of RLU for the LNCaP cells treated with androgen (+R1881) versus vehicle control (–R1881) with 1 and 5 μmol/L GWARJD10 were determined. SSAT transcriptional activity was significantly reduced with 5 μmol/L of GWARJD10 in the androgen treated cells. $N \geq 3$ per condition. $*P = 0.003$ compared to zero dose control. **(b)** Inhibition of induction of PSA: LNCaP cells in low androgen FIC4 medium were treated with drug GWARJD10 at 5 μmol/L (D) and/or androgen R1881 at 2 nmol/L (R). The conditions included: vehicle control (–R, –D), 5 μmol/L of GWARJD10 drug (–R, +D), androgen-stimulated (+R, –D), and androgen plus 5 μmol/L GWARJD10 (+R, +D). GWARJD10 significantly reduced PSA mRNA under the androgen-deprived condition ($P < 0.01$ for –R, –D compared to –R, +D at all timepoints). As expected, 2 nM androgen stimulation led to a significant increase in PSA mRNA ($P < 0.001$ for +R, –D compared to –R, –D at all timepoints). However, GWARJD10 did not affect PSA mRNA levels under the 2 nM androgen-stimulated condition, as no difference was observed at any timepoint for +R, –D compared to +R, +D. $N = 6$ per condition.

DISCUSSION

In the present study, we searched for small molecule inhibitors in two chemical libraries, a NCI Diversity Set [16] and a Life Chemicals Library [17] containing approximately 2,000 and 25,000 molecules, respectively, that can specifically block the interaction of activated AR with JunD. Twenty-two such inhibitors were selected from the screen and analyzed for their AR-LBD binding property. Eleven compounds that do not compete with androgen for binding to AR-LBD and have structures that are amenable to chemical synthesis were studied further in ROS and growth assays in prostate cancer cells. Three lead compounds, GWARJD07, GWARJD10, and GWARJD14, were markedly effective in blocking androgen-induced cellular ROS, as well as inhibiting growth of androgen-dependent LNCaP and its castrate-resistant variant C4-2 cells (Fig. 3). GWARJD10 was selected as the lead compound, based on its structure, lack of chemical anti-oxidant properties, and effect against ROS and growth as explained in the Results section, for further studies of the mechanism of action.

The rationale for seeking the agents that do *not* inhibit AR ligand-binding was based on the fact that current antiandrogens used in the clinic that generally target AR LBD, that is, compete with androgen for binding to AR-LBD, are only partially effective. Furthermore, there are reports that suggest an *agonist* potential of a major clinical anti-androgen that targets the AR-LBD [25–28], and/or the development of resistance of PCa to a more recent anti-androgen such as Enzalutamide through induction of mutations in the AR-LBD or activation of alternative pathways that leads to castrate-resistant PCa [29,30]. Recent evidence also shows the importance of inhibiting AR activation by a small molecule that binds to its N-terminus Domain (NTD) instead of the LBD at the C-terminus zone [31]. These inhibitors may block AR activation by inhibiting its interaction with other co-activators [31,32]. Mutational analysis have revealed that NTD is involved in transcriptional activity of AR and mutations in this region do not affect androgen-binding but abrogate AR transcriptional activity [33], therefore targeting AR NTD by small molecules may inhibit AR activity without affecting androgen-binding to AR [31].

Although we did not map the binding sites of these inhibitors in AR-JunD complex structure, the results from the AR ligand-binding competition assay showed that none of the selected inhibitors of AR-JunD interaction can effectively compete with androgen-binding to the AR-LBD domain. Furthermore, the results from quantitative analysis of PSA expression, where lead compound GWARJD10 had no effect on androgen

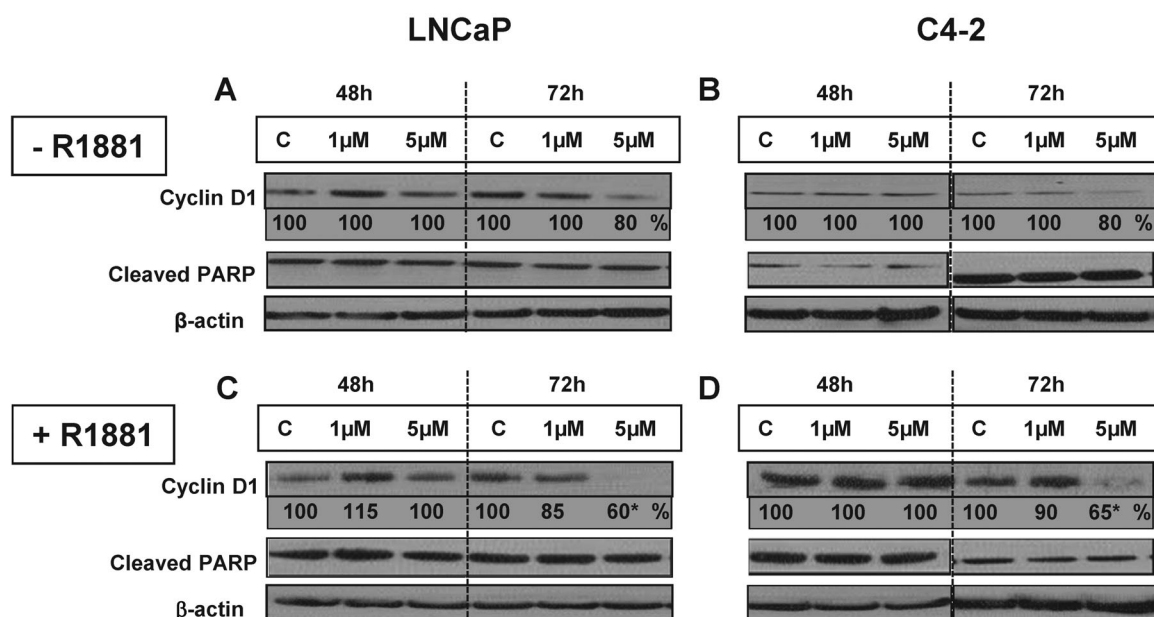


Fig. 6. Effect of GWARJD10 on apoptosis marker (Cleaved PARP) and cell cycle marker (Cyclin D1) in androgen-dependent LNCaP cells and its castrate-resistant variant C4-2 cells. LNCaP or C4-2 cells were treated with (+) or without (–) 2 nmol/l androgen R1881 and zero (C), 1 or 5 μmol/l of lead compound GWARJD10 for 48 and 72 hr. Cells were then harvested and protein levels were determined by Western blot analysis. β-actin was used as loading control and normalization. Percentage of each protein band compared to C (%) at each timepoint was calculated after normalizing each band against β-actin. Representative blots are shown. The level of Cleaved PARP (89 kDa) did not change under treatment conditions. Cyclin D1 (37 kDa) level was markedly reduced in R1881 treated LNCaP and C4-2 cells after 72 hr. The western blots were run three times for cell lysates from three to four different experiments per condition with similar results. Statistical analysis of bands across the experiments showed significantly lower (*) average cyclinD1 of 65% in LNCaP cells ($P = 0.001$, $N = 3$) and 71% in C4-2 cells ($P = 0.05$, $N = 4$) compared to control.

activation of AR to induce PSA, yet did reduce AR transcriptional activity under androgen deprived conditions, indicate that the inhibition of AR transcriptional activity by this compound is likely through its binding to other sites of AR, for example, NTD or DBD, and not to the LBD. The reduction in PSA expression as well as the lower transcriptional activity of SSAT promoter by this compound are not due to lower expression or degradation of AR or JunD proteins, since we did not observe any changes in AR or JunD protein levels or their stability in western blots under the conditions that the studies were performed (data not shown). Therefore, we concluded that these inhibitors bind to other domains in AR and likely at the JunD-AR interface. We are currently investigating the binding location of GWARJD10 in AR-JunD complex using structural biology approaches.

After identification of GWARJD10 as the lead compound, we used the IC_{50} concentration (5 μmol/l) of this compound to study its effect on the disruption of AR-JunD interaction in LNCaP cells using a Proximity Ligation Assay (PLA). The results from the PLA studies demonstrate a significant disruption of AR and JunD interaction in androgen treated LNCaP cells by GWARJD10 (Fig. 4c). We have shown before that JunD

is activated and translocated to the nucleus in androgen treated LNCaP cells [14]. Concerted activation and translocation of both transcription factors to the nucleus may lead to the activation of genes regulated by both AR and JunD, or the AR-JunD complex may transcribe a new set of genes that is not activated by either of them alone. For example, we have shown before that activated AR with JunD is involved in the induction of the *ssat* gene expression, yielding overexpression of SSAT enzyme that causes significant generation of ROS in the presence of androgen in LNCaP cells [14,15]. The ability of 5 μmol/l GWARJD10 to block the androgen-induced *ssat* promoter transcriptional activity (Fig. 5) provides further evidence of the ability of this compound to inhibit AR-JunD interaction. Thus, blocking the interaction between the AR and JunD may down regulate the expression of genes that are involved in growth and ROS generation in PCa cells.

Studies on the mechanism of GWARJD10 in growth inhibition of LNCaP and C4-2 cells show that this compound at 5 μmol/l is not cytotoxic and probably does not affect apoptosis since the level of cleaved PARP, a marker of apoptosis, did not change under different conditions (Fig. 6; panels a–d). We also

looked at other apoptosis markers such as Caspases 3 and 7, and likewise did not observe changes in the level of expression of these markers (data not shown). Since cell kill or apoptosis did not appear to be a factor in growth inhibitory effects of GWARJD10 under our conditions, we looked at a cell cycle marker cyclin D1. Cyclin D1 is a cell cycle regulator protein. Its over-expression has been shown to be associated with prostate cancer cell metastasis to the bone [34]. Additional support for potential role of cyclin D1 in androgen-independent PCa was derived from both in vitro and in vivo data [35]. Our data indicate that the growth inhibitory effect of this compound is at least in part due to its effect on cyclin D1 and related cell cycle regulatory gene expression. GWARJD10 at IC_{50} $5 \mu\text{mol/l}$ caused approximately 20% decrease in cyclin D1 in androgen-dependent LNCaP and castrate-resistant C4-2 cells (Fig. 6a, b). Interestingly, the effect of GWARJD10 on cyclin D1 was more pronounced under high ROS conditions. There are reports that explored the role of ROS on cyclin D1 in different conditions [36,37]. These reports showed that ROS degrade cyclin D1 and thereby prevent cell proliferation. We have observed that at 2 nmol/l R1881, which is the growth inhibitory concentration of R1881 and maximum ROS production [8], the expression of cyclinD1 is dramatically reduced (compare Fig. 6a with Fig. 6c in control lanes). GWARJD10 caused an additional 40% decrease in cyclin D1 under these conditions (Fig. 6c, d), and also blocked ROS production by approximately 40% (Fig. 3b) at $5 \mu\text{mol/l}$. The results from this study suggest that GWARJD10 at $5 \mu\text{mol/l}$ inhibits cell growth by reducing cyclin D1 expression in prostate cancer cells and also reduces ROS production in these cells by blocking AR-JunD interaction and thus, preventing *ssat* gene expression. Therefore, we believe this class of compounds will inhibit PCa growth, and could prevent progression to CRPCa that may be due to accumulation of ROS through this pathway. Further studies are needed to address how GWARJD10 concurrently causes the reduction in cyclin D1 and ROS.

In vivo studies with GWARJD10 are currently underway to elucidate the effectiveness of this compound in inhibition of growth and progression of prostate cancer.

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